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No Association Between Polymorphisms in the Human Dopamine D_3 and D_4 Receptors Genes and Alcoholism

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The human dopamine D_2 receptor gene (DRD_2) has received considerable attention for the past several years as a potential candidate that may affect susceptibility to alcoholism. The association studies that compared the frequencies of alleles of DRD_2 gene between alcoholics and control groups have produced equivocal results. Dopamine D_3 and D_4 receptor genes (DRD_3 and DRD_4) are in the same class as DRD_2 but with different pharmacological properties. We have used relative risk and haplotype relative risk approaches to test associations between alleles of DRD_3 and DRD_4 genes and alcoholism. For relative risk studies 162 probands from multiple incidence alcoholic families have been compared to 89 psychiatrically normal controls. Haplotype relative risk approaches have used 29 alcoholic probands in which both parents were available for genotyping. The Bal I restriction enzyme site in DRD_3 and tandem repeat (VNTR) in DRD_4 genes polymorphisms were used to genotype the above samples. The results of relative risk approaches for both DRD_3 and DRD_4 genes were negative for comparisons of alcoholics and subtypes of alcoholics with normal controls. Haplotype relative risk approaches also were negative for both genes. These results suggest that any role played by these receptors may account for only part of the variation in susceptibility to alcoholism. Am. J. Med. Genet. 74:281–285, 1997. © 1997 Wiley-Liss, Inc.

KEY WORDS: DRD_3; DRD_4; alcoholism; relative risk; haplotype relative risk

INTRODUCTION

The human dopamine D_2 receptor gene (DRD_2) has received considerable attention for the past several years as a potential candidate that may affect susceptibility to alcoholism. The association studies that compared the frequencies of alleles of DRD_2 gene between alcoholics and control groups have produced equivocal results [Blum et al., 1990; Parsian et al., 1991; Amadeo et al., 1993; Bolos et al., 1990; Gelernter et al., 1991; Turner et al., 1992]. Dopamine D_3 and D_4 receptors genes (DRD_3 and DRD_4) are in the same class as DRD_2 or D_2-like receptors but with different pharmacological properties. A polymorphism in the DRD_3 gene is present in the first exon and results in a glycine to serine substitution in the amino terminal of the amino acid sequence. Therefore, this polymorphism may represent a functional difference in the DRD_3 gene product. This polymorphism is detected by Bal I restriction enzyme digestion of genomic DNA amplified by the polymerase chain reaction [PCR, Lannfelt et al., 1992]. Interest in the DRD_3 gene as a possible candidate gene for schizophrenia has been generated in part by its tissue distribution of expression, which is more localized to limbic regions and its reported interactions with atypical neuroleptics [Sokoloff et al., 1990]. Hence, there have been several association studies between schizophrenia and increased homozygosity at the DRD_3 gene locus with equivocal results [Crocq et al., 1992; Jonsson et al., 1993; Mant et al., 1994]. In the case of alcoholism, there have been only two reports of lack of association with DRD_3 polymorphism [Adamson et al., 1995a; Gorwood et al., 1995].

The cloning of DRD_4 was reported by Van Tol et al. [1991] and showed that the pharmacological characteristics of this receptor resemble that of DRD_2 and DRD_3, but its affinity for clozapine is an order of magnitude higher. Later, the same group reported that clozapine binds to the DRD_4 with an affinity 10 times higher than the DRD_2 and DRD_3 receptors [Van Tol et al., 1992]. They also reported the existence of a 48-base

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pair repeat in the putative third cytoplasmic loop of the gene. Expression of the cDNA for three cloned receptor variants showed different properties for the 7-repeat form and the 2- and 4-repeat forms regarding clozapine and spiperone binding [Van Tol et al., 1992]. Following the characterization of the 48-base repeat in the DRD_4 gene by PCR in unrelated individuals by Lichter et al., [1993], a number of association studies with psychiatric disorders, especially schizophrenia and bipolar affective disorder, appeared in the literature most of which were negative [Daniels et al., 1994; Lim et al., 1994].

In the case of alcoholism, there are three reports of association studies with DRD_4 polymorphism. The first report was by George et al., [1993] who compared 72 severely affected chronic alcoholics to the reported normals in the literature. The alcoholics demonstrated a greater prevalence of the three- and six-repeat alleles than had been reported in normals ($P<0.005$). It is difficult to interpret these results since there is no information regarding the normal controls. The second report was by Adamson et al. [1995b] who compared 113 Finnish alcoholics most of whom showed early age of onset, impulsivity, and antisocial behavior to 113 Finnish controls without alcohol, drug abuse, and major mental illness. There was no association between DRD_4 alleles and alcoholism. There was also no association between CSF concentrations of the monoamine metabolites and DRD_4 genotypes. The third report was by Muramatsu et al. [1996], who compared 80 alcoholics with point mutation in the aldehyde dehydrogenase 2 gene ($ALDH_2{}^2$ allele) with 100 alcoholics without $ALDH_2{}^2$ allele and 144 controls in Japanese population. The frequency of the five repeat allele was three times more in alcoholics with $ALDH_2{}^2$ allele than alcoholics without the mutation and controls ($P<0.001$).

More recently, Ebstein et al. [1996] reported an association between the 7-repeat allele of DRD_4 and Novelty Seeking scores in a sample of 124 normal adult male and female volunteers. The presence of the 7-repeat allele had a significant main effect on the dependent variables, Novelty Seeking whereas ethnicity, sex, and age did not have significant effects. It is very interesting and important that this association study was replicated by Benjamin et al. [1996] in both population and family samples. A sample of 315 subjects were genotyped for the DRD_4 repeat polymorphism and the genotypes were divided into two groups. Alleles with 2 to 5 repeats were considered short (S), whereas alleles with 6 to 8 repeats were considered long (L). The scores for Extraversion were significantly higher ($P = 0.001$) and the scores for Conscientiousness were significantly lower ($P = 0.03$) in L than in S subjects. The estimated TPQ-Novelty seeking scores were associated with the long alleles ($P = 0.002$). Benjamin et al. [1996] also reported that similar results were obtained when genotypes were stratified according to Ebstein et al. [1996] by the presence or absence of the 7-repeat allele. In association study within pedigrees, 60 sib-pairs in which one sib had an L and one sib had an S genotype were used. The results showed that the L siblings scored significantly higher on NEO-PI-R Extraversion, lower on Conscientiousness and higher on estimated TPQ-Novelty Seeking than the S siblings from the same family [Benjamin et al., 1996]. It was concluded that these results indicate that the association is due to genetic transmission rather than population stratification.

TABLE I. Allele Frequencies for DRD_3 Gene in Alcoholic and Normal Control Groups

	Alleles[a]		
Group	1	2	Total
Alcoholics	220	104	324
Normal Controls	134	44	178

[a]Comparing all alleles together, the differences between the two groups were not significant [$\chi^2 = 3.01$, $P = 0.099 \pm 0.002$ (S.E.)].

In most of the association studies, relative risk (RR, case-control) comparison is used. This approach always raises questions regarding the ethnicity and population stratification. The alternative approach is to construct a control group using parental alleles that are not transmitted (haplotype relative risk, HRR). We have used both approaches to test associations between alleles of the DRD_3 and DRD_4 genes and alcoholism.

MATERIALS AND METHODS

Subjects

The subjects for these studies include probands of multiple incidence alcoholism pedigrees and psychiatric normal controls. The identification, ascertainment, and evaluation of these groups have been detailed in previous publications [Parsian et al., 1991; Parsian and Todd, 1994; Suarez et al., 1994]. For relative risk (RR, case-control) comparisons, the alcoholic group consisted of 162 probands (117 males and 45 females) who met the modified Feighner [1972] criteria for alcoholism [Parsian et al., 1991]. The alcoholics were classified into type I and type II according to Cloninger's criteria [1987]. Type I alcoholism occurs in both men and women, has a later age of onset (after age 25), has a less severe course, and has little or no antisocial behavior. Type II alcoholism is found mainly in men, has early

TABLE II. Allele Frequencies for DRD_4 Gene in Alcoholic and Normal Control Groups

	Allele (number of repeats)[a]							
Group	8R	7R	6R	5R	4R	3R	2R	Total
Alcoholics[b]	0	70	3	4	191	17	37	322
Normal controls	1	35	1	2	108	8	21	176

[a]Allele numbers represent the number of 48-base repeats; Size = 800–500bp.
[b]Comparing all alleles together, the differences between the two groups were not significant [$\chi^2 = 2.43$, $P = 0.912 \pm 0.002$ (S.E.)].

age of onset (before age 25), is more severe, and is associated with antisocial behavior. The normal control group consisted of 89 unrelated individuals (46 males and 43 females) who met no DSM-III-R criteria for affective disorders, alcoholism, schizophrenia, or other psychotic or drug use disorders. For the haplotype relative risk (HRR) analyses we also genotyped the parents of 29 alcoholic probands who were part of the case-control study. All subjects are white caucasian of Western European extraction. Self-defined ethnicity of the alcoholic probands and normal control group were determined by asking participants to identify the ethnic group of their four grandparents. The ethnicity of both groups, by percentage, was almost identical.

Genotyping

High molecular weight genomic DNA was extracted from whole blood or transformed cell lines. The dopamine D_3 receptor gene region of interest was amplified and genotyped using PCR followed by restriction enzyme digestion with MscI, which is the isoschizomer of Bal I [Lannfelt et al., 1992]. The complete procedure with modifications has been detailed in a previous publication [Parsian et al., 1995]. The dopamine D_4 receptor region containing the 48-base repeat was amplified by PCR using the primers sequences published by Shaikh et al. [1993]. The PCR cocktail included 50 mM KCl, 10 mM Tris.HCl, 1.5 mM $MgCl_2$, 10% dimethylsulfoxide, 200 μM dNTP substituting 7-deazaguanosine for dGTP, 10 pmol each primer with the sense primer [α-^{32}P] ATP end labeled, and 50 ng genomic DNA. The PCR reaction was denatured for 5 minutes at 94°C followed by 35 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 40 seconds with a final extension of 72°C for 5 minutes. PCR cycling was performed with a Perkin-Elmer-Cetus 9600 thermocycler (Norwalk, CT). The PCR products were separated on a 5% denaturing polyacrylamide gel ran at 30 W for 4 hours and detected via exposure to X-ray film for 16 hours at room temperature. All genotypes were interpreted independently by two trained observers without knowledge of the patient diagnosis.

Statistical and Genetic Analyses

In relative risk analyses, allele frequency comparisons were performed using the χ^2 method. For simultaneous comparisons of the frequencies of multiple alleles (where many values are less than five) the χ^2 probability distribution was estimated using Monte Carlo simulation [Roff and Bentzen, 1989] as implemented by a computer program developed by George Carmody (Carleton University, Ottawa, Ontario). The advantages of this computational approach is that χ^2 simulations with estimated standard errors can be completed in a matter of minutes without collapsing cells with small numbers of observations. Haplotype relative risk (HRR) analyses were performed as described by Falk and Rubinstein [1987] except that individual alleles, rather than genotypes, were contrasted [Parsian et al., 1995].

TABLE III. Allele Frequencies for DRD_3 Gene in Subtypes of Alcoholics and Normal Controls

	Alleles[a]		
Group	1	2	Total
Type II alcoholics	128	58	186
Type I alcoholics	83	41	124
Normal controls	134	44	178

[a]Comparing all alleles together, the differences between the three groups were not significant (Type II vs. NC, $\chi^2 = 1.88$, $P = 0.204 \pm 0.003$; Type I vs. NC, $\chi^2 = 2.51$, $P = 0.119 \pm 0.002$; Type II vs. Type I, $\chi^2 = 0.121$, $P = 0.806 \pm 0.003$).

RESULTS

Tables I and II present the allele frequencies for the DRD_3 and DRD_4 genes for both alcoholic and normal control groups. In the case of DRD_4, the allele numbers represent the number of 48-base (VNTR) repeats. To compare all alleles together Monte Carlo simulations of the χ^2 probability distribution were performed [Roff and Bentzen, 1989] using a computer program developed by Carmody (1995). For both DRD_3 and DRD_4 the overall comparisons between alcoholic and normal control groups were not significant [$\chi^2 = 3.01$, $P = 0.099 \pm 0.002$; $\chi^2 = 2.43$, $P = 0.912 \pm 0.002$ (S.E.), respectively]. These analyses are performed with 17,000 simulations as suggested for these sort of data by Carmody (1995). In the case of the DRD_3 gene, the frequency of allele number 2 was higher in alcoholics than normal controls (0.32 vs. 0.25) but the difference is not significant. However, the frequencies of DRD_4 alleles were almost identical in both alcoholics and normal controls. The alcoholics were categorized into type I and type II according to Cloninger's criteria [1987]. From 162 alcoholics, 93 were type II, 62 were type I, and 7 could not be classified due to lack of cooperation. Tables III and IV represent the allele frequencies for DRD_3 and DRD_4 genes in subtypes of alcoholics and normal controls. For both genes, the differences between allele frequencies in three groups, namely, type I and type II alcoholics and normal controls, were not

TABLE IV. Allele Frequencies for DRD_4 Gene in Subtypes of Alcoholics and Normal Controls

	Allele (number of repeats)[a]							
Group	8R	7R	6R	5R	4R	3R	2R	Total
Type II alcoholics	0	34	3	2	116	11	20	186
Type I alcoholics	0	33	0	2	66	5	16	122
Normal controls	1	35	1	2	108	8	21	176

[a]Comparing all alleles together, the differences between the three groups were not significant (Type II vs. NC, $\chi^2 = 2.52$, $P = 0.927 \pm 0.00$; Type I vs. NC, $\chi^2 = 3.90$, $P = 0.762 \pm 0.003$; Type II vs. Type I, $\chi^2 = 6.42$, $P = 0.270 \pm 0.003$).

TABLE V. Haplotype Relative Risk Analysis for DRD_3 Gene in Alcoholic Families

Allele number	Transmitted (n = 56)	Non-transmitted[a] (n = 56)
1	41	36
2	15	20

[a]Total transmitted versus non-transmitted (n = 112); $\chi^2 = 1.04$, $P = 0.413 \pm 0.004$.

significant (DRD_3:type II vs. NC, $\chi^2 = 1.88$, $P = 0.204 \pm 0.003$; type I vs NC, $\chi^2 = 2.51$, $P = 0.119 \pm 0.002$; type II vs. type I, $\chi^2 = 0.121$, $P = 0.806 \pm 0.003$; DRD_4: type II vs. NC, $\chi^2 = 2.52$, $P = 0.927 \pm 0.002$; type I vs. NC, $\chi^2 = 3.9$, $P = 0.762 \pm 0.003$; type II vs. type I, $\chi^2 = 6.42$, $P = 0.27 \pm 0.003$).

As mentioned above, in relative risk (case-control) analyses there are concerns regarding the matching strategies and population stratification. Falk and Rubinstein [1987] proposed a new approach (haplotype relative risk) in which the proband and the parents are genotyped for any gene or marker. The non-transmitted parental alleles are used as controls. In haplotype relative risk analyses, alleles were used for both DRD_3 and DRD_4 genes in a sample of 29 probands and their parents who were informative. The summary of these analyses for DRD_3 and DRD_4 are presented by alleles in tables V and VI, respectively. The differences between total transmitted alleles vs. non-transmitted alleles for both genes were not significant (DRD_3: $\chi^2 = 1.04$, $P = 0.413 \pm 0.004$; DRD_4: $\chi^2 = 4.903$, $P = 0.27 \pm 0.004$). However, allele number 1 for DRD_3 and allele number 5 for DRD_4 were more transmitted than non-transmitted, but the differences were not significant. It is important to note that the results of both statistical analyses are similar.

DISCUSSION

We compared the allele frequencies of the DRD_3 gene between alcoholics, who were classified into type I and type II, to normal controls. The results of all comparisons, including the alcoholic subtypes, were negative (Tables I and III). Therefore, our results are similar to the Adamson et al. [1995a] and Gorwood et al. [1995] studies. The main difference is that both our alcoholic and control groups are well characterized and matched for ethnicity. In Gorwood et al. [1995], three different samples with three different ascertainment criteria were mixed but the results were negative. We performed the same analyses for the DRD_4 gene polymorphism and the overall allele comparisons between the four groups were negative (Tables II and IV). However, George et al. [1993] reported that the frequencies of DRD_4 alleles with three and six repeats were significantly higher in alcoholics than reported controls ($P<0.005$). The frequencies of these two alleles in our alcoholics and normal controls are 0.053, 0.009 and 0.045, 0.005, respectively. These differences between alleles frequencies among groups are not significant. Therefore, the results of George et al. [1993] could be due to chance and population stratification. Adamson et al. [1995] also reported that no association was detected between a particular DRD_4 allele and alcoholism in a Finnish population.

TABLE VI. Haplotype Relative Risk Analysis for DRD_4 Gene in Alcoholic Families

Allele number	Transmitted (n = 58)	Non-transmitted[a] (n = 58)
7R	16	18
5R	1	0
4R	39	33
3R	0	2
2R	2	5

[a]Total transmitted versus non-transmitted (n = 116); $\chi^2 = 4.903$, $P = 0.27 \pm 0.004$.

Now, it has been well established that in case-control studies, population stratification will produce false positive results. Therefore, identification of a proper control group is essential. It is obvious that ascertainment of such a control group in a mixed population is very difficult. An alternative approach is the haplotype relative risk (HRR) method. We have also used this method for both DRD_3 and DRD_4 data (Tables V and VI). The differences between the overall alleles transmitted versus non-transmitted for DRD_3 and DRD_4 genes in alcoholic families were not statistically significant. These results support our case-control findings. Therefore, we conclude that the two functional polymorphisms of DRD_3 and DRD_4 are not associated with alcoholism in our samples.

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